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Dopamine counteracts octopamine signaling in a neural circuit mediating food response in *C. elegans*

Joseph Culotti and Hubert H. M. Van Tol

Corresponding author: Satoshi Suo, Mount Sinai Hospital

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 March 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all the referees find your work interesting, and are broadly supportive of publication. However, all three highlight a number of concerns that would need to be addressed in a revised version of the manuscript before we could consider publication.

I hope you understand if I do not go into detail of all the points raised, but one major concern (highlighted by both reviewers 1 and 3) is that the contribution of serotonin - which, like dopamine, is involved in the sensing of food availability - has not been investigated. Similarly, the question as to functional interplay between the mechanism you identify here, and the previously identified regulation of octopamine by TGFbeta signalling, needs to be addressed more directly. One further concern regards your analysis as to which dopamine receptors are involved in mediating these effects: both referees 2 and 3 require further clarification on this point.

In the light of the referees' positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Therefore, acceptance of your paper will depend on your ability to fully answer the points raised by the referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Suo and colleagues had previously reported that starvation induces cAMP response element binding protein (CREB) gene expression in SIA neurons and that this phenotype is dependent on octopamine signaling through the SER-3 octopaminergic receptor on SIA neurons. In this manuscript, the authors demonstrate that dopamine signaling counteracts octopamine induced CREB gene expression in SIA neurons. Using dopamine and octopamine synthesis deficient mutants, they demonstrate that results obtained through exogenously added monoamine transmitters reflect endogenous regulatory mechanisms. By examining various receptor mutants, they demonstrate that dopamine receptors encoded by dop-2 and dop-3 account for a significant portion of dopaminergic effects on octopamine dependent CREB gene expression. To further understand the regulatory circuitry between dopaminergic and octopaminergic signals they reconstitute dop-2 and dop-3 receptors in SIA and octopamine-synthesizing RIC neurons showing that expression in these neurons is sufficient to mediate the effects of dopamine on expression pattern of their CREB reporter in SIA neurons.

This study is interesting as it investigates how food-related changes become manifested in changes in neural signaling cascades and understanding these regulatory cascades at the level of functional circuitry with single neuron resolution level. The authors provide an interesting discussion comparing the regulatory framework revealed by their study and a potential counterpart in mammalian brain. The experiments and the text are presented in a straight-forward manner and the presented the data generally support the stated conclusions. The manuscript, however, is too narrowly focused on the interaction of dopamine and octopamine signaling pathways and minor revisions to the text are needed for a more accurate presentation. Specifically:

1) As in dopamine, serotonin is thought to be a signal of food availability. The authors should at least investigate whether serotonin signaling counteracts the effects of octopamine signaling on CREB gene expression.

2) As indicated by the authors in the discussion session, data from Greer et al indicates that reduction in DAF-7/TGF-b signaling relieves inhibition of octopamine signaling from RIC neurons. Thus, it appears that dopamine and TGF-b signaling cascades represent at least two modalities through which animals can perceive alterations in food availability and regulate octopamine signaling. The authors should therefore investigate the hierarchical relationship of these two pathways by examining whether daf-7 mutants on food exhibit CREB expression in SIA neurons. Also, the introduction currently states "However, the way in which absence of food induces the octopamine signal remains unknown" (page 4). This statement obviously ignores the indicated manuscript. Similarly, on page 16 (first paragraph of discussion), the authors write: "cessation of dopamine signaling in the absence of food is the primary mechanism of up-regulating octopamine signaling". Given the manuscript by Greer et al, lack of direct experiments with daf-7 mutants, and partial effects noted throughout the text, this claim is not justified.

3) Page 7: The authors write: "These results demonstrate that, in well-fed animals, exogenous dopamine suppresses exogenous octopamine-mediated CREB activation in the SIA neurons". However, as the authors accurately point out at various other places in the text, the dopaminergic signal appears to mediate mechanical sensation of food presence rather than a state of being well-fed.

4) Finally, the authors provide no data indicating the physiological significance of CREB activation in SIA neurons, thus, for the moment, this neuron specific gene expression only serves as a reporter. The authors should either provide experimental evidence for the relevance of this expression or temper down their writing by acknowledging this issue in a more upfront fashion in the text.

Referee #2 (Remarks to the Author):

In this study the authors have characterized a very interesting antagonism between the neuromodulators dopamine and octopamine. They taken advantage of the relatively "simple" nervous system of C. elegans to do so and their work adds to our growing understanding how of

how multiple signals cooperate to control signaling levels.

Overall this is a very nice study and a well-written manuscript that is appropriate for publication in EMBO. I have no major criticisms.

Minor Points:

1) The authors may want to point out that dopamine (and octopamine?) acts humorally in C. elegans, and so direct synaptic contacts between the involved neurons is not necessarily required. Along this line, the authors may want to clarify whether there are synaptic connections between the dopaminergic neurons, RIC and/or SIA neurons.

2) On page 7 the authors wait until their discussion of Figure 2 to define cat-2, although cat-2 animals are used in Figure 1. They should be described earlier, given their inclusion in Figure 1.

3) I am confused by the rational presented on page 19. This line of argument needs to be clarified: "However, DOP-2 and DOP-3 are likely to be the only dopamine receptors working in the SIA neurons, since the response to exogenous dopamine was entirely dependent on the dop-2 and dop-3 genes." Immediately before this sentence, the authors specifically propose the existence of a yet uncharacterized novel dopamine receptor. This is based on the finding that the dop-2;dop-3 phenotype is not as strong as the cat-2 phenotype when spontaneous CREB activation is scored (which is a readout of endogenous signaling levels). As this experiment relates to endogenous signaling, isn't it likely a better indicator of endogenous physiological signaling than the result obtained with the addition of exogenous dopamine? I'm not sure why the authors use this as the basis to propose that DOP2 and DOP-3 are the only two DA repectors in SIA. Is it also possible that a D1-like receptor (DOP-1?) gets activated in SIA in response to the high levels of exogenous DA applied?
I might be missing the basis of their argument, further explanation in the text should easily rectify this.

4) Although dop-1 and dop-4 do not show a phenotype on their own, have the authors examined them in conjunction with loss of dop-3? This might be particularly interesting given (a) they site on page 21 that there is evidence in mammals that D1 and D2-type receptors antagonize eachother in the cholinergic neurons and (b) the work of Chase, Pepper and Koelle 2004 that showed that, while loss of dop-1 alone did not show an effect on basal slowing, when dop-1 dop-3 animals were tested it was seen that DOP-1 antagonizes DOP-3 in the same motor neurons. Importantly, the role of DOP-1 was only revealed in the absence of DOP-3.
While this experiment would be extremely interesting, in this reviewer's opinion, the major point of the paper (namely, the mechanism by which dopamine and octopamine antagonize each other to regulate downstream cellular effects such as transcription) does not depend on it.

5) The authors refer to a "three -neuron circuit" but it isn't clear that it's just three neurons. There are multiple dopaminergic neurons, and since dopamine can act at a distance in C. elegans, it could be coming from multiple sources. This may just be semantics, but perhaps there's a better way to name this model.

Referee #3 (Remarks to the Author):

Summary:
Suo et al. have performed a large set of elegant experiments to investigate the neural circuitry and signaling involved in an octopamine-dopamine regulated gene expression response to the presence/absence of food. Previously they found that starvation in C. elegans causes the expression of GFP driven by an engineered promoter containing multiple cre sites in a set of 4 neurons called SIAs; this effect was found to be mediated by octopamine released from 2 RIC neurons. Currently, they have shown that this neural response is suppressed by dopamine working through the DOP-2 and DOP-3 receptors and the Gprotein GOA-1. They suggest that dopamine is released in response to the mechanical stimulation caused by the presence of food (bacteria). The amount of data collected and thoroughness of their well thought-out experiments are impressive. Their results appear to be extremely robust and the implications will be of general interest to those who study the

signaling of neural circuits, particularly circuits mediating food-related responses. Other than a few minor concerns, the manuscript is an excellent contribution to the field.

Minor Concerns:

Has this been shown to be CREB dependent? Are all these effects blocked in a crh-1 KO? If this was in the 2006 paper it should be mentioned here.

Soaking is shown to induce a similar cre::GFP response; however, the authors show it is not dependent on either octopamine or dopamine. It is unclear the significance of this result. Either discuss these implications further or remove these data from the manuscript because they simple create confusion.

It is suggested that Sephadex bead treatment has been shown to activate DA signaling (eg. bottom of p.17). This is misleading because this has not been shown directly; although, there is evidence to suggest this might be the case. Re-word. Sephadex beads have been shown previously to rescue DA-dependent food-related behaviours in the absence of food.

"...considering that food intake per se has little effect on CREB activation..." no evidence is presented to suggest that food intake and CREB are not related. Please clarify: present supporting evidence.

Suo et al. suggest the possibility of another dopamine receptor (other than DOP-1,2,3, or 4) many have a role. Is there any genetic evidence for more dopamine receptors in C elegans genome? Also, if another dopamine receptor is involved in the suppression, wouldn't you expect dopamine treated dop-2;dop-3 mutants to have a slightly more suppressed response instead of an enhanced response? (Fig.3D first vs. third bar). Please discuss.

The abbreviation 'NC' is a confusing choice to represent 'no amine treatment'. Please consider changing it to a more intuitive abbreviation, such as 'NA' for 'no amine', or 'NT' for 'no treatment'.

The authors discuss a DAF-7 - TGFbeta pathway that has been implicated in starvation and how it may relate to the mechanism that they suggest; however, many starvation-related behaviours/responses in C. elegans are mediated/dependent on serotonin. Please discuss why this amine was not investigated, or why it may or may not take part in the cre::gfp response in the SIA neurons.

The authors speculate that the circuit is regulated by the balance between octopamine and dopamine; however some evidence in their results is contradictory to this-,perhaps they could suggest ways this might be tested.

Because cell-specific rescues of goa-1 were not performed the authors should soften their conclusions about the role of goa-1 specifically in SIA but not RIC. This should be discussed and reflected in the model. (Fig. 6)

The evidence supporting dop-3's role in RIC is very limited. Only two experiments implicates the possible role, and it is a partial effect and it is using the sephadex bead treatment (Fig.5I) which is likely a poor simulation of real food, and in Fig.5J, where they observed a very modest effect compared to the rest in the manuscript. Also, the octopamine treatment approach found null results (Fig. 5F), suggesting dop-3's role in RIC is inconclusive. The authors should be more cautious of including it in their model. On the other hand, the rest of the model seems well-supported with robust data.

Data that would benefit from further discussion:

(Fig. 1F first vs second bar and Fig. 2H - first vs. second bar) - if the octopamine/starvation response is caused by dopamine why does starvation still enhance the response in the dopamine deficient cat-2 mutants. Likely this is because cat-2 are not completely dopamine deficient. This could be mentioned in the text.

(Fig.1E third bar vs. Fig.1F third bar) - why doesn't exogenous dopamine in cat-2 look like wt worms (with exogenous dopamine)? The model proposed by Suo et al. suggests that this would greatly sway the balance of the circuit in the direction of dopamine, suppressing the octopamine induction of cre::gfp. Along the same lines, (Fig. 1E last bar vs Fig 1F last bar) - why doesn't O+D in cat-2 suppress the cre::gfp response to wild-type levels? Yet the cat-2;tbh-1 is wild-type (Fig.1H last bar). Please discuss in more detail.

(Fig.3G) - dop-1(vs100) mutants appear to have a smaller octopamine response. Why isn't this discussed? This may to be true for both alleles (ev748 and vs100) in the starvation response (Fir.3O+P), although much less of an effect. In fact, the dop-1 phenotype looks remarkably like dop-2;ceh-17p::dop-2(+) (Fig.5B) and dop-3;che-17p::dop-3(+) (Fig.5E); perhaps suggesting opposing roles. Perhaps dop-1 is responsible for the slight enhancement observed in the dop-2;dop-3 double mutants when treated with dopamine alone (Fig.3D third bar)?

1st Revision - authors' response 11 June 2009

ANSWERS TO REVIEWERS' COMMENTS:

I provide a point-by-point discussion of the changes made to the manuscript. The reviewers' comments are also presented here by copying and pasting the original.

Referee #1 (Remarks to the Author):

Suo and colleagues had previously reported that starvation induces cAMP response element binding protein (CREB) gene expression in SIA neurons and that this phenotype is dependent on octopamine signaling through the SER-3 octopaminergic receptor on SIA neurons. In this manuscript, the authors demonstrate that dopamine signaling counteracts octopamine induced CREB gene expression in SIA neurons. Using dopamine and octopamine synthesis deficient mutants, they demonstrate that results obtained through exogenously added monoamine transmitters reflect endogenous regulatory mechanisms. By examining various receptor mutants, they demonstrate that dopamine receptors encoded by dop-2 and dop-3 account for a significant portion of dopaminergic effects on octopamine dependent CREB gene expression. To further understand the regulatory circuitry between dopaminergic and octopaminergic signals they reconstitute dop-2 and dop-3 receptors in SIA and octopamine-synthesizing RIC neurons showing that expression in these neurons is sufficient to mediate the effects of dopamine on expression pattern of their CREB reporter in SIA neurons.

This study is interesting as it investigates how food-related changes become manifested in changes in neural signaling cascades and understanding these regulatory cascades at the level of functional circuitry with single neuron resolution level. The authors provide an interesting discussion comparing the regulatory framework revealed by their study and a potential counterpart in mammalian brain. The experiments and the text are presented in a straight-forward manner and the presented the data generally support the stated conclusions. The manuscript, however, is too narrowly focused on the interaction of dopamine and octopamine signaling pathways and minor revisions to the text are needed for a more accurate presentation. Specifically:

1) As in dopamine, serotonin is thought to be a signal of food availability. The authors should at least investigate whether serotonin signaling counteracts the effects of octopamine signaling on CREB gene expression.

REPLY: We have tested whether exogenous and endogenous serotonin counteracts the octopamine signaling that regulates CREB activation in the SIA neurons. We found that exogenous serotonin slightly suppresses exogenous octopamine-mediated CREB activation only at high concentrations, which are toxic to the animals. This suppression was also observed in the Gi/o-coupled serotonin receptor mutant ser-4, suggesting that it is not mediated by Gi/o-coupled receptor activity as was

observed for dopamine-mediated suppression. We also found that unlike dopamine-deficient cat-2 mutants, serotonin-deficient tph-1 mutants do not exhibit strong spontaneous CREB activation in the presence of food. These results suggest that serotonin does not play a major role in regulating octopamine-mediated CREB activation in the SIA neurons.

We present the serotonin data in Supplementary Figure S2 and describe the results in p.17 line 10-p.18 line 10.

2) As indicated by the authors in the discussion session, data from Greer et al indicates that reduction in DAF-7/TGF-b signaling relieves inhibition of octopamine signaling from RIC neurons. Thus, it appears that dopamine and TGF-b signaling cascades represent at least two modalities through which animals can perceive alterations in food availability and regulate octopamine signaling. The authors should therefore investigate the hierarchical relationship of these two pathways by examining whether daf-7 mutants on food exhibit CREB expression in SIA neurons. Also, the introduction currently states "However, the way in which absence of food induces the octopamine signal remains unknown" (page 4). This statement obviously ignores the indicated manuscript. Similarly, on page 16 (first paragraph of discussion), the authors write: "cessation of dopamine signaling in the absence of food is the primary mechanism of up-regulating octopamine signaling". Given the manuscript by Greer et al, lack of direct experiments with daf-7 mutants, and partial effects noted throughout the text, this claim is not justified.

REPLY: According to Greer et al., DAF-7 works on DAF-1 receptor in the RIM and/or RIC neurons, which in turn suppresses DAF-3 in the RIM and/or RIC neurons. Furthermore, decreased expression of DAF-7 in the absence of food induces activation of DAF-3. We have tested daf-7 and daf-1 mutants and found that these mutants exhibit significant but only slight spontaneous CREB activation in the presence of food, suggesting that the DAF-7 pathway plays a minor role in the regulation of CREB in the SIA neurons. We also tested daf-3 mutants and found that daf-3 mutants respond normally to the absence of food. This result suggests that, even in the absence of DAF-7-mediated regulation, dopamine signaling alone can regulate octopamine signaling in response to food.

We present these data in Figure 6 and describe the results in p.18 line 11-p.19 line 11.

We changed the sentence "However, the way in which absence of food induces the octopamine signal remains unknown" to "It is reported that octopamine signaling works downstream of the DAF-7 TGF signaling pathway in C. elegans (Greer et al, 2008). However, it is unknown whether any other signaling pathway regulates octopamine signaling in this animal." (p.4 line 14-17).

We changed "cessation of dopamine signaling in the absence of food is the primary mechanism of up-regulating octopamine signaling" to "the cessation of dopamine signaling in the absence of food is an important mechanism for up-regulating octopamine signaling" (p.20 line 6-7).

3) Page 7: The authors write: "These results demonstrate that, in well-fed animals, exogenous dopamine suppresses exogenous octopamine-mediated CREB activation in the SIA neurons". However, as the authors accurately point out at various other places in the text, the dopaminergic signal appears to mediate mechanical sensation of food presence rather than a state of being well-fed.

REPLY: We removed "in well-fed animals" from the sentence (p.7 line 7-9).

4) Finally, the authors provide no data indicating the physiological significance of CREB activation in SIA neurons, thus, for the moment, this neuron specific gene expression only serves as a reporter. The authors should either provide experimental evidence for the relevance of this expression or temper down their writing by acknowledging this issue in a more upfront fashion in the text.

REPLY: We agree that the physiological function of CREB activation in the SIA neurons is unknown and it is worthwhile to acknowledge that fact. We stated it upfront in the Introduction (p.4 line 12-14).

Referee #2 (Remarks to the Author):

In this study the authors have characterized a very interesting antagonism between the

neuromodulators dopamine and octopamine. They taken advantage of the relatively "simple" nervous system of C. elegans to do so and their work adds to our growing understanding how of how multiple signals cooperate to control signaling levels.

Overall this is a very nice study and a well-written manuscript that is appropriate for publication in EMBO. I have no major criticisms.

Minor Points:

1) The authors may want to point out that dopamine (and octopamine?) acts humorally in C. elegans, and so direct synaptic contacts between the involved neurons is not necessarily required. Along this line, the authors may want to clarify whether there are synaptic connections between the dopaminergic neurons, RIC and/or SIA neurons.

REPLY: In the Introduction we state that dopamine acts humorally and therefore direct synaptic connection is not required (p.5 line 2-7). We also stated that the CEP class of dopaminergic neurons is pre-synaptic to the RIC and SIA neurons in the same part.

2) On page 7 the authors wait until their discussion of Figure 2 to define cat-2, although cat-2 animals are used in Figure 1. They should be described earlier, given their inclusion in Figure 1.

REPLY: We agree that the order of appearance in the figures does not correspond well with the text. Instead of changing the order of appearance in the test, we re-organized Figures 1 and 2. We removed the parts presenting cat-2, tbh-1, and cat-2;tbh-1 from Figure 1 and now present them in Figure 2.

3) I am confused by the rational presented on page 19. This line of argument needs to be clarified: "However, DOP-2 and DOP-3 are likely to be the only dopamine receptors working in the SIA neurons, since the response to exogenous dopamine was entirely dependent on the dop-2 and dop-3 genes." Immediately before this sentence, the authors specifically propose the existence of a yet uncharacterized novel dopamine receptor. This is based on the finding that the dop-2;dop-3 phenotype is not as strong as the cat-2 phenotype when spontaneous CREB activation is scored (which is a readout of endogenous signaling levels). As this experiment relates to endogenous signaling, isn't it likely a better indicator of endogenous physiological signaling than the result obtained with the addition of exogenous dopamine? I'm not sure why the authors use this as the basis to propose that DOP2 and DOP-3 are the only two DA repectors in SIA. Is it also possible that a D1-like receptor (DOP-1?) gets activated in SIA in response to the high levels of exogenous DA applied?

I might be missing the basis of their argument, further explanation in the text should easily rectify this.

REPLY: Our experiments suggest that exogenous dopamine works directly on the SIA neurons and the exogenous dopamine response is dependent only on DOP-2 and DOP-3. We therefore concluded that DOP-2 and DOP-3 are the only dopamine receptors that suppress octopamine signaling in the SIA neurons. However, we agree that endogenous dopamine may have some function on the SIA neurons (in addition to the effect on the RIC neurons) that is not replicated by exogenous dopamine.

We also realized that the dop-2 and dop-3 alleles used in this study are not experimentally determined to be null alleles. Even though the deletions in these alleles remove at least one transmembrane domain of the receptors, there is a possibility that these alleles are not null. Therefore, the observation that dop-2;dop-3 double mutants shows weaker spontaneous CREB activation than cat-2 mutants could be attributable to residual activity of dop-2 and dop-3. Thus, "Our results predict the existence of a novel dopamine receptor" might be an overstatement. We removed the paragraph discussing the possible involvement of another dopamine receptor from the manuscript. We also mention the possibility that the dop-2 and dop-3 alleles are not null in p.12 line 1-3 and p.14 line 13-17.

With respect to the possibility that the D1-like receptor activates CREB in response to exogenous dopamine, we have conducted experiments to address this issue. Please see the reply to the next comment.

4) Although dop-1 and dop-4 do not show a phenotype on their own, have the authors examined

them in conjunction with loss of dop-3? This might be particularly interesting given (a) they site on page 21 that there is evidence in mammals that D1 and D2-type receptors antagonize eachother in the cholinergic neurons and (b) the work of Chase, Pepper and Koelle 2004 that showed that, while loss of dop-1 alone did not show an effect on basal slowing, when dop-1 dop-3 animals were tested it was seen that DOP-1 antagonizes DOP-3 in the same motor neurons. Importantly, the role of DOP-1 was only revealed in the absence of DOP-3.

While this experiment would be extremely interesting, in this reviewer's opinion, the major point of the paper (namely, the mechanism by which dopamine and octopamine antagonize eachother to regulate downstream cellular effects such as transcription) does not depend on it.

REPLY: We tested dop-1;dop-2;dop-3 and dop-2;dop-3;dop-4 triple mutants to examine whether dop-1 or dop-4 exhibits an opposing function to dop-2 and dop-3. We found that dop-1 has no influence on CREB activation of dop-2;dop-3 double mutants. On the other hand, dop-4 mutations mildly suppressed spontaneous CREB activation of dop-2;dop-3 double mutants. dop-4 mutations also suppressed dopamine-mediated CREB activation observed for dop-2;dop-3 double mutants. These results suggest that DOP-4, but not DOP-1, opposes DOP-2 and DOP-3 and positively regulates CREB.

We present these data in Supplementary Figure S1 and describe the results in p.12 line 18 - p.13 line 9.

5) The authors refer to a "three -neuron circuit" but it isn't clear that it's just three neurons. There are multiple dopaminergic neurons, and since dopamine can act at a distance in C. elegans, it could be coming from multiple sources. This may just be semantics, but perhaps there's a better way to name this model.

REPLY: We agree that it is not just three neurons. There are three classes (8 in total) of dopaminergic neurons and we do not know which one is important in the regulation of CREB. Moreover, there are two octopaminergic RIC neurons and four SIA neurons. We changed from "three-neuron" to "three-neuron-type" throughout the text. The title was also changed to remove "three-neuron circuit".

Referee #3 (Remarks to the Author):

Summary:
Suo et al. have performed a large set of elegant experiments to investigate the neural circuitry and signaling involved in an octopamine-dopamine regulated gene expression response to the presence/absence of food. Previously they found that starvation in C. elegans causes the expression of GFP driven by an engineered promoter containing multiple cre sites in a set of 4 neurons called SIAs; this effect was found to be mediated by octopamine released from 2 RIC neurons. Currently, they have shown that this neural response is suppressed by dopamine working through the DOP-2 and DOP-3 receptors and the Gprotein GOA-1. They suggest that dopamine is released in response to the mechanical stimulation caused by the presence of food (bacteria). The amount of data collected and thoroughness of their well thought-out experiments are impressive. Their results appear to be extremely robust and the implications will be of general interest to those who study the signaling of neural circuits, particularly circuits mediating food-related responses. Other than a few minor concerns, the manuscript is an excellent contribution to the field.

Minor Concerns:

Has this been shown to be CREB dependent? Are all these effects blocked in a crh-1 KO? If this was in the 2006 paper it should be mentioned here.

REPLY: In the 2006 paper, we show that CREB activation in the absence of food requires crh-1. We state it in this manuscript in p.6 line 7-8.

Soaking is shown to induce a similar cre::GFP response; however, the authors show it is not dependent on either octopamine or dopamine. It is unclear the significance of this result. Either discuss these implications further or remove these data from the manuscript because they simple create confusion.

REPLY: The finding itself that dopamine is not involved in soaking-mediated CREB activation is new and we believe that it is worthwhile mentioning even though it is not directly related to the major point of the paper. In addition, soaking-mediated CREB activation indicates that the SIA neurons exist and are capable of activating CREB in each mutant strain. Especially in the cases of cat-2;tbh-1 double mutants and dop-3 mutants carrying ceh-17/tbh-1::dop-3, normal soaking responses in these strains suggest that the decreased CREB activation (in the presence or absence of food or Sephadex) observed in these strains is unlikely to be attributable to developmental defects that disrupt generation or function of the SIA neurons in general. We discuss these implications on p.9 line 10-17 and p.16 line 20-22.

It is suggested that Sephadex bead treatment has been shown to activate DA signaling (eg. bottom of p.17). This is misleading because this has not been shown directly; although, there is evidence to suggest this might be the case. Re-word. Sephadex beads have been shown previously to rescue DA-dependent food-related behaviours in the absence of food.

REPLY: The sentence "we used sephadex beads (SX) that mimic the tactile attribute of the bacterial food source and thereby activate the mechanosensory dopaminergic neurons even in the absence of ingestion or chemosensation of food" was changed to "we used the Sephadex beads (SX). It was shown previously that the Sephadex beads induce a dopamine-dependent behavioral change presumably by mimicking the tactile attribute of the bacterial food source without providing nutritional or chemosensory cues associated with bacteria" (p.8 line 5-9).
The sentence "Furthermore, sephadex treatment, which is shown to activate dopamine signaling," was changed to "Furthermore, Sephadex bead treatment, which is shown to mediate a food-related behavior in a dopamine-dependent manner" (p.21 line 16-17).

"...considering that food intake per se has little effect on CREB activation..." no evidence is presented to suggest that food intake and CREB are not related. Please clarify: present supporting evidence.

REPLY: We agree that this was an overstatement. We changed the sentence "considering that food intake per se has little effect on CREB activation" to "considering that well-fed cat-2 animals exhibit CREB activation" (p. 23 line 6-7).

Suo et al. suggest the possibility of another dopamine receptor (other than DOP-1,2,3, or 4) many have a role. Is there any genetic evidence for more dopamine receptors in C elegans genome? Also, if another dopamine receptor is involved in the suppression, wouldn't you expect dopamine treated dop-2;dop-3 mutants to have a slightly more suppressed response instead of an enhanced response? (Fig.3D first vs. third bar). Please discuss.

REPLY: Our previous sequence analyses suggested that there are no other G protein-coupled receptors that show strong homology to known dopamine receptors. We state this on p.10 line 16-18.
We realized that the alleles of dop-2 and dop-3 used in this study have not been experimentally determined to be null alleles. Therefore, the observation that dop-2;dop-3 double mutants shows weaker spontaneous CREB activation than cat-2 mutants could be attributable to residual activity of dop-2 and dop-3. Thus, "Our results predict the existence of a novel dopamine receptor" might be an overstatement. We removed the paragraph discussing the possible involvement of another dopamine receptor from the manuscript. We also mention the possibility that the dop-2 and dop-3 alleles are not null in p.12 line 1-3 and p.14 line 13-17.
With respect to the exogenous dopamine-mediated slight enhancement, we tested dop-1 and dop-4 in the dop-2;dop-3 background and found that dop-4 is responsible for this response. This result suggests that dop-4 opposes dop-2 and dop-3 and positively regulates CREB activation and that this is the mechanism for dopamine-mediated activation. We present these data in Supplementary Figure S1 and describe the results in p.12 line 18 - p.13 line 9.

The abbreviation 'NC' is a confusing choice to represent 'no amine treatment'. Please consider changing it to a more intuitive abbreviation, such as 'NA' for 'no amine', or 'NT' for 'no treatment'.

REPLY: We changed "NC" to "NA" throughout the manuscript.

The authors discuss a DAF-7 - TGFbeta pathway that has been implicated in starvation and how it may relate to the mechanism that they suggest; however, many starvation-related behaviours/responses in C. elegans are mediated/dependent on serotonin. Please discuss why this amine was not investigated, or why it may or may not take part in the cre::gfp response in the SIA neurons.

REPLY: We have tested whether exogenous and endogenous serotonin counteracts octopamine signaling in the regulation of CREB activation in the SIA neurons. We found that exogenous serotonin slightly suppresses exogenous octopamine-mediated CREB activation only at a high concentration, which is toxic to the animals. This suppression was also observed in the Gi/o-coupled serotonin receptor mutant ser-4, suggesting that it is not mediated by Gi/o-coupled receptor activity differing in this respect from dopamine-mediated suppression. We also found that, unlike the dopamine-deficient cat-2 mutant, the serotonin-deficient tph-1 mutants do not exhibit strong spontaneous CREB activation in the presence of food. These results suggest that serotonin does not play a major role in regulating octopamine-mediated CREB activation in the SIA neurons.
We present these data in Supplementary Figure S2 and describe the results on p.17 line 10 - p.18 line 10.

The authors speculate that the circuit is regulated by the balance between octopamine and dopamine; however some evidence in their results is contradictory to this-,perhaps they could suggest ways this might be tested.

REPLY: When discussing "the balance between octopamine and dopamine", we were referring to the regulation in the SIA neurons alone but not the entire circuit. We acknowledge that use of the term "balance" may be a vague way to describe the observed regulation. We removed the term and re-worded to say "It is likely that activation of CREB in the SIA neurons is determined by the relative strength of the dopamine and octopamine signals." We also made some changes in the paragraph to clarify the point, which is that activation of CREB in the SIA neurons is determined by the relative strength of octopamine and dopamine signal and not simply by whether each signal is present or not (p.20 line 20 - p.21 line 11).

Because cell-specific rescues of goa-1 were not performed the authors should soften their conclusions about the role of goa-1 specifically in SIA but not RIC. This should be discussed and reflected in the model. (Fig. 6)

REPLY: In the previous study, we found that goa-1 works downstream of octopamine presumably in the SIA neurons. As pointed out, since cell-specific rescues were not done, it is unknown whether goa-1 works in the RIC neurons or not. However, it is likely to work in the RIC neurons since goa-1 is expressed in all the neurons and DOP-3 is shown to couple to Gi/o. We discussed this in the Discussion (p.22 line 18-21) and included goa-1 in the RIC neurons of Figure 7 (formerly Figure 6). Since the function of goa-1 in the RIC neurons is not experimentally determined, question marks were added beside goa-1.

The evidence supporting dop-3's role in RIC is very limited. Only two experiments implicates the possible role, and it is a partial effect and it is using the sephadex bead treatment (Fig.5I) which is likely a poor simulation of real food, and in Fig.5J, where they observed a very modest effect compared to the rest in the manuscript. Also, the octopamine treatment approach found null results (Fig. 5F), suggesting dop-3's role in RIC is inconclusive. The authors should be more cautious of including it in their model. On the other hand, the rest of the model seems well-supported with robust data.

REPLY: We agree that it is possible that the Sephadex beads do not completely mimic the tactile stimulation of food. However, the sephadex response defect in dop-3 strongly suggests that the Sephadex beads suppress octopamine-dependent CREB activation through dopamine signaling (because DOP-3 is shown to be a dopamine receptor) (discussed on p.22 line 1-11). Suppression of this defect by expression of dop-3 in the RIC neurons clearly indicates that dop-3 functions in the RIC neurons and plays a role in dopamine signaling.
As pointed out, dop-3 expression in the RIC neurons did not rescue the response to exogenous dopamine (Figure 5F). However, this observation does not contradict the model we presented. Our

results indicate that exogenous dopamine and octopamine work directly on the SIA neurons and that loss of endogenous octopamine has no influence on the dopamine-mediated suppression of exogenous octopamine-mediated CREB activation (since tbh-1 responds normally to exogenous dopamine (Figure 2M)). Therefore, suppression of octopamine release from the RIC neuron by DOP-3 should have no influence on the response to exogenous dopamine, which is consistent with the observed results.

We also tested the influence of the DAF-7 TGF signaling pathway on the CREB activation in the SIA neurons, since the DAF-7 pathway was suggested to regulate octopamine signal from the RIC neurons. We found that food can regulate CREB activation even in the absence of the regulation by the DAF-7 signaling. Although there could be another pathway that regulates the RIC neurons, the observation that well-fed cat-2 animals exhibit CREB activation also suggests that the octopamine signal is active whenever the suppression by dopamine is absent. Taken together, it is likely that dopamine regulates the function of the RIC neurons.

We acknowledge that the role of dop-3 in the RIC neurons should be discussed more thoroughly. We therefore included the points made above in the Discussion (p.22 line 12 - p.23 line 13).

Data that would benefit from further discussion:

(Fig. 1F first vs second bar and Fig. 2H - first vs. second bar) - if the octopamine/starvation response is caused by dopamine why does starvation still enhance the response in the dopamine deficient cat-2 mutants. Likely this is because cat-2 are not completely dopamine deficient. This could be mentioned in the text.

REPLY: We mention this observation in the text and describe the possible involvement of residual dopamine (p.8 line 21 - p.9 line 2).

(Fig.1E third bar vs. Fig.1F third bar) - why doesn't exogenous dopamine in cat-2 look like wt worms (with exogenous dopamine)? The model proposed by Suo et al. suggests that this would greatly sway the balance of the circuit in the direction of dopamine, suppressing the octopamine induction of cre::gfp. Along the same lines, (Fig. 1E last bar vs Fig 1F last bar) - why doesn't O+D in cat-2 suppress the cre::gfp response to wild-type levels? Yet the cat-2;tbh-1 is wild-type (Fig.1H last bar). Please discuss in more detail.

REPLY: In these amine treatment experiments, animals are grown in normal growth media that does not contain octopamine or dopamine. Therefore, prior to dopamine treatment, cat-2 mutants should have already expressed GFP in the SIA neurons. It is possible that 6 hr of incubation in dopamine was not sufficient for the preexisting GFP to be degraded and become undetectable. We discuss this in p.9 line 21- p.10 line 5.

(Fig.3G) - dop-1(vs100) mutants appear to have a smaller octopamine response. Why isn't this discussed? This may to be true for both alleles (ev748 and vs100) in the starvation response (Fir.3O+P), although much less of an effect. In fact, the dop-1 phenotype looks remarkably like dop-2;ceh-17p::dop-2(+) (Fig.5B) and dop-3;che-17p::dop-3(+) (Fig.5E); perhaps suggesting opposing roles. Perhaps dop-1 is responsible for the slight enhancement observed in the dop-2;dop-3 double mutants when treated with dopamine alone (Fig.3D third bar)?

REPLY: We mention the smaller octopamine response of dop-1(vs100) mutants (p.12 line 11-14). However, given that dop-1(ev748) mutants do not show this defect, we stated that the role of dop-1 in this response is "unclear".

We also tested dop-1;dop-2;dop-3 triple mutants, and found that dop-1 is not responsible for the slight enhancement of CREB activation by dopamine. Instead we found that dop-4 suppresses spontaneous CREB activation and the dopamine-mediated enhancement observed in dop-2;dop-3 double mutants, suggesting an opposing role for dop-4 but not dop-1. We now present these data in Supplementary Figure S1 and describe the results in p.12 line 18 - p.13 line 9.

Because of inclusion of new data and additional discussion, a part of Materials and Method was removed from the main text and included as Supplementary Materials and Method, so that the manuscript is less than 55,000 characters.